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Research Article

A new genus and new species of freshwater Chaetonotidae (Gastrotricha: Chaetonotida) from Brazil with phylogenetic position inferred from nuclear and mitochondrial DNA sequences

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Most studies of South American freshwater Gastrotricha have focused on the inland waters of Brazil including São Paulo State, Mato Grosso do Sul State, and Pará State. In this paper, we describe a new genus and species, *Cephalionotus kisielewskii* gen. et. sp. nov. (Chaetonotida, Chaetonotidae), from Minas Gerais State using morphological data and DNA sequence data (18S rDNA and COI genes). The new genus contains a single psammic species collected from high altitude streams. *Cephalionotus* gen. nov. exhibits a set of autapomorphic characters that easily distinguish it from all other genera of Chaetonotida: extremely large cephalion and hypostomium that completely cover the dorsal and ventral sides of the head, respectively; a single pair of pleurae; peculiar shape and arrangement of dorsal and ventral scales in the posterior trunk region; one paired, very large, keeled scale at the ventral posterior end; and very long adhesive tubes divided into three parts. Affinity of *Cephalionotus kisielewskii* gen. et sp. nov. with other Chaetonotida (Paucitubulatina) is discussed from morphological and molecular perspectives.

Key words: inland waters, meiofauna, Neotropical biogeographic region, phylogeny, psammic, systematics

Introduction

Gastrotricha is a constant and important component of benthic microinvertebrate communities in marine and freshwater habitats (Balsamo & Todaro, 2002; Balsamo, d'Hondt, Kisielewski, & Pierboni, 2008; Balsamo, d'Hondt, Pierboni, & Grilli, 2009; Balsamo, Guidi, & d'Hondt, 2013; Balsamo et al., 2015). Although many gastrotrich species are common, and occasionally abundant, in freshwater habitats, the taxon is still poorly known, possibly due to the microscopic size, fragile body, complex morphology of its representatives and low number of specialists on gastrotrich taxonomy (Balsamo & Todaro, 2002; Balsamo et al., 2008; Hochberg & Litvaitis, 2000a).

Approximately 820 species of gastrotrichs are known (Araújo & Hochberg, 2016; Araújo, Atherton, & Hochberg, 2015; Balsamo et al., 2015; Garraffoni & Melchior, 2015; Garraffoni, Di Domenico, & Hochberg, 2016; Kieneke, Schmidt-Rhaesa, & Hochberg, 2015; Kolicka, Jankowska, & Kotwicki, 2015; Todaro, Dal Zotto, & Leasi, 2015a; Todaro, Perissinotto, & Bownes, 2015b), traditionally grouped into two orders: Macrotrichida Remane, 1925 [Rao & Clausen, 1970] and Chaetonotida Remane, 1925 [Rao & Clausen, 1970] (but see Kieneke, Riemann, & Ahlrichs, 2008). The order Chaetonotida, suborder Paucitubulatina d'Hondt, 1971, includes more than 465 species with a tenpin-shaped body, sometimes living in marine or brackish water, but mostly in freshwater habitats. The largest family is the cosmopolitan Chaetonotidae Gosse, 1864 [sensu Leasi & Todaro, 2008], which currently includes more than 335 species in 14 genera and two subfamilies

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(seven genera are exclusively freshwater – *Arenotus* Kisielowski, 1987, *Bifidochaetus* Kolicka & Kisielowski, in press, *Fluxiderma* d’Hondt, 1974, *Lepidochaetus* Kisielowski, 1991, *Polymerurus* Remane, 1927a, *Rhomballichthys* Schwank, 1990 and *Undula* Kisielowski, 1991; two are exclusively marine – *Caudichthyidium* Schwank, 1990 and *Halichaetonotus* Remane, 1936; and five are both freshwater and marine – *Aspidiophorus* (Voigt, 1902), *Chaetonotus* Ehrenberg, 1830, *Heterolepidoderma* Remane, 1927a, *Ichthyidium* Ehrenberg, 1830 and *Lepidodermella* Blake, 1933) (Balsamo *et al.*, 2009; Balsamo, Grilli, Guidi, & d’Hondt, 2014; Hummon & Todaro, 2010; Kolicka *et al.* in press; Todaro, 2016).

Here, we formally describe a new species of freshwater Gastrotricha from Brazil belonging to a new genus previously reported by Araújo, Alcântara, and Garraffoni (2013) as an unnamed taxon ('gen. nov., sp. nov.') and Araújo, Checon, and Garraffoni (2016) as 'Chaetonotidae sp.' We also examine the affinity of this new taxon with the other genera of Chaetonotida (Paucitubulatina) based on nuclear (18S rDNA) and mitochondrial DNA (COI) sequences. Finally, this study advances the effort to increase taxonomic and biogeographic knowledge of Brazilian freshwater Gastrotricha. Prior to this study, the freshwater fauna of South America was known to comprise 97 species from Argentina, Brazil, Guyana, Paraguay, and Uruguay (Cordero, 1918; d’Hondt, Pourriot, & Rougier, 2006; d’Hondt, Pourriot, Rougier, & Guiral, 2005; Daday, 1905; Grosso, 1973a, 1973b; Grosso, 1975, 1976, 1982; Grosso & Drahg, 1983, 1984, 1986, 1991) with 58 of these species present in Brazil (Araújo *et al.*, 2013; Garraffoni & Melchior, 2015; Garraffoni, Araújo, Lourenço, & Balsamo, 2010; Kånneby, Todaro, & Jondelius, 2013; Kisielowski, 1987, 1991; Todaro *et al.*, 2012b).

Materials and methods

Sampling

Samples of the upper sediment layer were collected with a manual corer from five distinct stations in Jequitinhonha Basin, State of Minas Gerais, South-eastern Brazil: Site 1: Araçuaí River (sandy and rocky substrate), 17°57'17"S, 43°14'47"W; Sites 2, 3, 5: Preto River (sandy/muddy/rocky substrate), 18°08'13"S, 43°20'13"W; 18°06'59"S, 43°20'26"W; 18°02'50"S–43°22'26"W; Site 4: Veado Pool (sandy substrate), 18°06'51"S, 43°20'39"W; Site 6: Éguas Stream (rocky substrate), 18°08'11"S, 43°20'30"W; Site 7: Jequitinhonha River (sandy substrate), 18°06'51"S, 43°31'14"W; Sites 8, 9: Inhacica River (sandy/muddy/rocky substrate), 17°50'26"S, 43°45'10"W; 17°47'85"S, 43°38'43"W (Fig. 1).

Gastrotrichs were detected by sorting small amounts of sediment poured into Petri dishes under a Leica EZ4

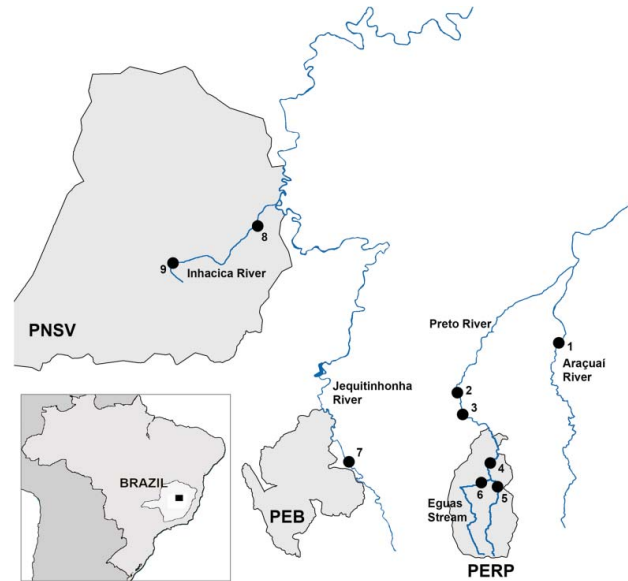


Fig. 1. Localities in the State of Minas Gerais (Brazil) where *Cephalionotus kisielowskii* gen. et sp. nov. was found. PNSV: Parque Nacional da Sempre Viva (Sempre Viva National Park), PEB: Parque Estadual do Biribiri (Biribiri State Park), PERP: Parque Estadual do Rio Preto (Rio Preto State Park). Numbers represent the sampling sites in this study, listed in the Materials and methods.

stereomicroscope. The animals were isolated alive, anaesthetized with 2% MgCl₂, mounted singly on glass slides, and digitally documented under a Zeiss Axio Imager M2 light microscope equipped with differential interference contrast (DIC) and an AxioCam MRC5 digital video camera. The video files are available from the first author upon request.

Scanning electron microscopy

For scanning electronic microscopy (SEM) analysis, after sorting, the specimens were fixed in 10% borax-buffered formalin, rinsed in 0.1 M PBS (pH 7.4), dehydrated with a graded ethanol series, treated with HMDS (hexamethyldisilazane) (Hochberg & Litvaitis, 2000b), mounted on aluminium stubs, and sputter coated with gold-palladium. Observations were carried out under a Philips 515 scanning electron microscope, at the University of Urbino (Italy).

DNA extraction and amplification

DNA was extracted from a single specimen of *Cephalionotus kisielowskii* gen. et sp. nov. using a QIAamp DNA Micro Kit (Qiagen), following the manufacturer's instructions. PCR amplifications was performed in a reaction mixture containing 3 µL of genomic DNA, 12.5 µL of Taq PCR Master Mix (Qiagen), 8.7 µL of water, and

Table 1. Primer sequences and PCR regimes used for 18S rDNA and COI mtDNA amplification and sequencing.

Primers & PCR regimes		
18S primers	Primer sequence (5'→3')	Reference
S30	GCTTGTCTCAAAGATTAAGCC	Norén and Jondelius (1999)
S30R	CTTCGGACCTCTGACTTTTCG	This study
PCR S30/S30R	94°C for 5 min, 40× (94°C for 30 s, 52.5°C for 30 s, 72°C for 60 s), 72°C for 7 min	
1801	GATCTATTTTGTGGTTTTTCGG	This study
1806	CCTTGTTACGACTTTTACTTCCTC	Norén and Jondelius (1999)
PCR 1801/1806	94°C for 5 min, 40× (94°C for 30 s, 52.5°C for 30 s, 72°C for 60 s), 72°C for 7 min	
COI primers		
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer, Black, Hoeh, Lutz, and Vrijenhoek (1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
PCR LCO1490/ HCO2198	94°C for 5 min, 40× (94°C for 30 s, 46°C for 30 s, 72°C for 40 s), 72°C for 7 min	

0.4 µL (4 pmol) of specific primers. The primer sequences and PCR regimes are indicated in Table 1. The amplification products were analysed by electrophoresis in 1% agarose gels containing SYBR® Green (Life Technologies), and the expected bands were excised and then purified using a QIAquick Gel Extraction Kit (Qiagen). The DNA fragments were sequenced using BigDye Terminator reactions in a 3730XL DNA Analyzer (Applied Biosystems) at the LaCTAD laboratory (Campinas, Brazil). The 18S rDNA (1716 base pairs; GenBank accession no.: KX159486) and COI mtDNA (640 base pairs; GenBank accession no.: KX159487) partial DNA sequences were deposited in GenBank.

Sequences, alignments, and data analyses

18S rDNA and COI mtDNA sequences were aligned separately with Mafft v.7.215 using the L-INS-I approach (Katoh & Standley, 2013). The best-fit substitution model was determined with jModelTest 2.1.4 (Darriba, Taboada, Doallo, & Posada, 2012). The concatenated dataset (18S rDNA and COI mtDNA) was analysed using maximum likelihood (ML) and Bayesian inference methods (BA). ML analysis using RAxML (Stamatakis, Hoover, & Rougemont, 2008) was run with a GTRCAT model with 1000 bootstrap replicates. BA analysis was done using MrBayes v.3.2.3 (Ronquist & Huelsenbeck, 2003) using two different runs with four chains each for a maximum of 20 million generations (sampled every 500 generations). Best-fit evolutionary model selected was GTR + G. The analysis was stopped when the two runs reached convergence (average standard deviation of split frequencies under 0.01). Convergence and estimated sample size (ESS) were verified using TRACER v.1.5, and 10% of each run was

discarded as burn-in. Both ML and BA analyses were performed using the CIPRES Science Gateway, San Diego Supercomputer Center (Miller, Pfeiffer, & Schwartz, 2010).

All formally described species of the order Chaetonotida of which sequences of both 18S rDNA and COI mtDNA are available in GenBank were included in the present analysis (45 species: 35 Chaetonotidae, six Dasydytidae Daday, 1905, two Neogosseidae Remane, 1927a and two Xenotrichulidae Remane, 1927b). The species and respective GenBank accession numbers are listed in Table 2. Although Balsamo et al. (2009) considered the genus *Lepidochaetus* Kisielowski, 1991 a subgenus of *Chaetonotus*, we decided to keep it as a distinct genus to avoid conflict with the species name inserted in GenBank (Table 2). Furthermore, in the analysis by Kånneby et al. (2013) the genus *Chaetonotus* appeared as polyphyletic whereas the taxon *Lepidochaetus* as monophyletic.

Kånneby et al. (2013) and Kånneby and Todaro (2015) found Dasydytidae and Neogosseidae nested within Chaetonotidae, therefore we decided to use Xenotrichulidae (*Draculiciteria tessellata* [Renaud Mornant, 1968], *Xenotrichula intermedia* Remane, 1934) as outgroups.

Results

Taxonomic account

Phylum Gastrotricha Metschnikoff, 1865
 Order Chaetonotida Remane, 1925 [Rao & Clausen, 1970]
 Suborder Paucitubulatina d'Hondt, 1971
 Family Chaetonotidae Gosse, 1864 [*sensu* Leasi & Todaro, 2008]

Table 2. Taxa included in this study, with GenBank accession numbers of 18S rDNA and COI mtDNA sequences.

Species	18S	COI	Reference
Family Chaetonotidae			
<i>Arenotus strixinoi</i> Kisielowski, 1987	JQ798537	JQ798677	Kanneby et al. (2013)
<i>Aspidiophorus paramediterraneus</i> Hummon, 1974	JQ798538	JQ798678	Kanneby et al. (2013)
<i>Aspidiophorus polystictos</i> Balsamo & Todaro, 1987	JQ798598	JQ798727	Kanneby et al. (2013)
<i>Aspidiophorus tentaculatus</i> Wilke, 1954	JQ798553	JQ798690	Kanneby et al. (2013)
<i>Aspidiophorus tetrachaetus</i> Kisielowski, 1986	JN185505	JN185576	Kanneby et al. (2012)
<i>Chaetonotus acanthodes</i> Stokes, 1887	JQ798585	JQ798716	Kanneby et al. (2013)
<i>Chaetonotus aemilianus</i> Balsamo, 1978	JQ798556	JQ798693	Kanneby et al. (2013)
<i>Chaetonotus daphnes</i> Balsamo & Todaro, 1995	JQ798549	JQ798687	Kanneby et al. (2013)
<i>Chaetonotus</i> cf. <i>dispar</i> Wilke, 1954	JQ798561	JQ798696	Kanneby et al. (2013)
<i>Chaetonotus heideri</i> Brehm, 1917	JQ798547	JQ798685	Kanneby et al. (2013)
<i>Chaetonotus heteracanthus</i> Remane, 1927	JQ798543	JQ798681	Kanneby et al. (2013)
<i>Chaetonotus</i> cf. <i>hystrix</i> Metschnikoff, 1865	JQ798603	JQ798732	Kanneby et al. (2013)
<i>Chaetonotus</i> cf. <i>laroides</i> Marcolongo, 1910	JQ798602	JQ798731	Kanneby et al. (2013)
<i>Chaetonotus</i> cf. <i>maximus</i> Ehrenberg, 1838	JQ798574	JQ798706	Kanneby et al. (2013)
<i>Chaetonotus microchaetus</i> Preobrajenskaja, 1926	JQ798583	JQ798714	Kanneby et al. (2013)
<i>Chaetonotus neptuni</i> Wilke, 1954	JQ798595	JQ798724	Kanneby et al. (2013)
<i>Chaetonotus</i> cf. <i>novenarius</i> Greuter, 1917	JQ798566	JQ798699	Kanneby et al. (2013)
<i>Chaetonotus</i> cf. <i>oculifer</i> Kisielowski, 1981	JQ798576	JQ798708	Kanneby et al. (2013)
<i>Chaetonotus polyspinosus</i> Greuter, 1917	JQ798563	JQ798698	Kanneby et al. (2013)
<i>Chaetonotus schultzei</i> Metschnikoff, 1865	JQ798596	JQ798725	Kanneby et al. (2013)
<i>Chaetonotus similis</i> Zelinka, 1889	JQ798578	JQ798710	Kanneby et al. (2013)
<i>Chaetonotus</i> cf. <i>sphagnophilus</i> Kisielowski, 1981	JQ798604	JQ798733	Kanneby et al. (2013)
<i>Halichaetonotus aculifer</i> (Gerlach, 1953)	JQ798550	JQ798688	Kanneby et al. (2013)
<i>Halichaetonotus paradoxus</i> (Remane, 1927)	JQ798599	JQ798728	Kanneby et al. (2013)
<i>Heterolepidoderma acidophilum</i> Kanneby et al., 2012	JN185462	JN185543	Kanneby et al. (2012)
<i>Heterolepidoderma macrops</i> Kisielowski, 1981	JN185469	JN185548	Kanneby et al. (2012)
<i>Heterolepidoderma ocellatum</i> (Metschnikoff, 1865)	JN185476	JN185555	Kanneby et al. (2012)
<i>Ichthyidium squamigerum</i> Balsamo & Fregni, 1995	JQ798607	JQ798736	Kanneby et al. (2013)
<i>Ichthyidium skandicum</i> Kanneby, Todaro & Jondelius, 2009	JQ798573	JQ798705	Kanneby et al. (2013)
<i>Lepidochaetus brasiliense</i> Kisielowski, 1991	JN185495	JN185568	Kanneby et al. (2013)
<i>Lepidochaetus zelinkai</i> (Grünspan, 1908)	JN185486	JN185564	Kanneby et al. (2013)
<i>Lepidodermella intermedia</i> Kanneby et al., 2012	JN185468	JN185547	Kanneby et al. (2012)
<i>Lepidodermella squamata</i> (Dujardin, 1841)	JN185479	JN185558	Kanneby et al. (2012)
<i>Polymerurus nodicaudus</i> (Voigt, 1901)	JN185502	JN185573	Kanneby et al. (2012)
<i>Polymerurus rhomboides</i> (Stokes, 1887)	JN185493	JN185567	Kanneby et al. (2012)
Family Dasydytidae			
<i>Dasydytes carvalhoae</i> Kisielowski, 1991	JQ798570	JQ798702	Kanneby et al. (2013)
<i>Dasydytes elongatus</i> Kisielowski, 1991	JQ798568	JQ798700	Kanneby et al. (2013)
<i>Dasydytes papaveroi</i> Kisielowski, 1991	JQ798571	JQ798703	Kanneby et al. (2013)
<i>Ornamentula paraënsis</i> Kisielowski, 1991	JQ798562	JQ798697	Kanneby et al. (2013)
<i>Stylochaeta fusiformis</i> (Spencer, 1890)	JN185471	JN185550	Kanneby et al. (2012)
<i>Stylochaeta scirtetica</i> Brunson 1950	JN185492	JN185566	Kanneby et al. (2012)
Family Neogosseidae			
<i>Kijanebalola devestiva</i> Todaro et al. 2013	KR822112	KR822120	Kanneby and Todaro (2015)
<i>Neogosseia acanthocolla</i> Kisielowski, 1991	KR822114	KR822121	Kanneby and Todaro (2015)
Family Xenotrichulidae			
<i>Draculiciteria tessellata</i> (Renaud-Mornant, 1968)	JN185470	JN185549	Kanneby et al. (2012)
<i>Xenotrichula intermedia</i> Remane, 1934	JF357664	JF432048	Todaro et al. (2011)

Emended diagnosis of the family Chaetonotidae

Paucitubulatina with body 60–770 μm long. Head rounded, cephalic plates occasionally present, very large and very close to each other, fully covering the head (*Cephalionotus*); present as a dorsofrontal cephalion, one or two pairs of lateral pleurae, and a ventral hypostomium; head tentacles lacking, but small cephalic papillae occasionally present. Caudal furca bearing a pair of terminal adhesive tubes; adhesive tubes occasionally absent (*Undula*). Cephalic ciliature in one or two paired lateral tufts, or, occasionally, one paired undulated transverse row (*Undula*). Ventral locomotory ciliature usually in two longitudinal bands extending along the whole body. Ciliary bands may occasionally be interrupted. When present, one to three (usually two) paired dorsal sensory bristles generally on scales with a double keel or (rarely) double spine. Body at least partially covered with spined or keeled scales, pedunculated scales or scales bearing lamellae, occasionally with a layer of smooth cuticle or scales without spines (*Arenotus*, *Caudichthyidium*, *Ichthyidium*). Ventral intercalary area covered with simple, spined or pedunculated scales, or naked; at least one pair of intercalary terminal scale generally present, occasionally much larger than the others. Mouth terminal or subterminal, ring-shaped, often with simple or articulated rod-like structures. Four to five paired longitudinal muscles; helicoidal muscles extending posteriorly to the pharyngo-intestinal junction; circular muscles restricted to the pharyngeal region; dorso-ventral muscles occasionally present, in splanchnic position, along the intestinal region (*Polymerurus*). Parthenogenic, aberrant spermatozoa often present at a post-parthenogenic phase, with functions unknown. Eggs maturing dorsally. Freshwater, marine and brackish-water. Benthic, periphytic, and occasionally semipelagic.

Two subfamilies. Chaetonotinae Gosse, 1864 [*sensu* Kisielowski, 1991] (type-subfamily) and Undulinae Kisielowski, 1991. The subfamily Chaetonotinae includes 13 genera: *Chaetonotus* Ehrenberg, 1830 (type-genus), *Arenotus* Kisielowski, 1987, *Aspidiophorus*, Voigt, 1903, *Bifidochaetus* Kolicka & Kisielowski, in press, *Caudichthyidium* Schwank, 1990, *Fluxiderma* d'Hondt, 1974, *Halichaetonotus* Remane, 1936, *Heterolepidoderma* Remane, 1926, *Ichthyidium* Ehrenberg, 1830, *Lepidochaetus* Kisielowski, 1991, *Lepidodermella* Blake, 1933, *Polymerurus* Remane, 1926, and *Rhomballichthys* Schwank, 1990. The subfamily Undulinae includes a single genus: *Undula* Kisielowski, 1991 (type-genus).

Cephalionotus gen. nov.

Diagnosis. Chaetonotinae with body length from 156 to 204 μm . Tenpin-shaped body, well-demarcated head, long and segmented furcal appendages. Rounded head with a large cephalion almost completely fused with the most anterior median scale and juxtaposed to the three posterior dorsal head scales; only one paired pleura. Hypostomium

as a large plate covering the whole head ventrally. Dorsal side of body covered with 7–9 alternating columns, each of 17–18 spineless scales, up to the last third of the body. Scales hexagonal or rounded, overlapping, mostly of constant size. Dorsal posterior trunk with 5 columns, each of 3–4 much larger spineless scales. Furca 41–42 μm in length, with a very short base covered with scales and two long, thin adhesive tubes divided into three parts (17, 10 and 7 μm in length, respectively). Two pairs of dorsal sensorial bristles. Ventral intercalary area covered with 3–5 alternating columns, each of 15–17 spineless, overlapping scales, rectangular in shape, and gradually increasing in size from anterior to posterior up to the last third of the body (U64). On the ventral posterior trunk, one paired large, keeled scale followed by one paired, long, thin, keeled scale and flanked by some spineless scales of varying sizes, the anteriormost much larger than the others. Mouth terminal. Pharynx with two strong bulbs connected by a short middle region without cuticular reinforcement.

Autapomorphies. Cephalion extremely wide, dorsally almost completely fused with the anteriormost median scale and juxtaposed to the first scale of the five median columns; one paired pleura; hypostomium extremely large, covering the whole head ventrally; furcal adhesive tubes divided into three parts. Distinctive scaling of the posterior trunk both dorsally and ventrally, featuring large spineless scales. Two paired, ventral, terminal, keeled scales.

Etymology. From the Greek ‘kephalē’ – head and Latin ‘notus’ – known, referring to the peculiar arrangement of the cephalic plates.

Cephalionotus kisielowskii sp. nov.

(Figs 2–19, Table 3)

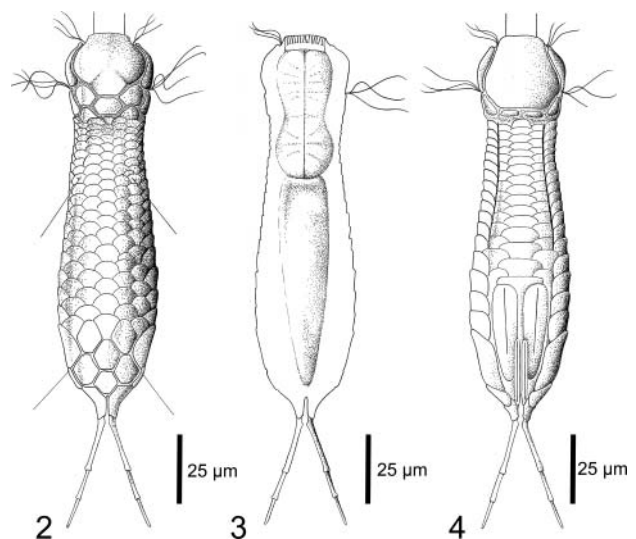


Fig. 2–4. Schematic drawing of *Cephalionotus kisielowskii* gen. et sp. nov. 2, dorsal view; 3, optical section; 4, ventral view: the two dark lines indicate the insertion of locomotory cilia. All drawings refer to the holotype specimen.

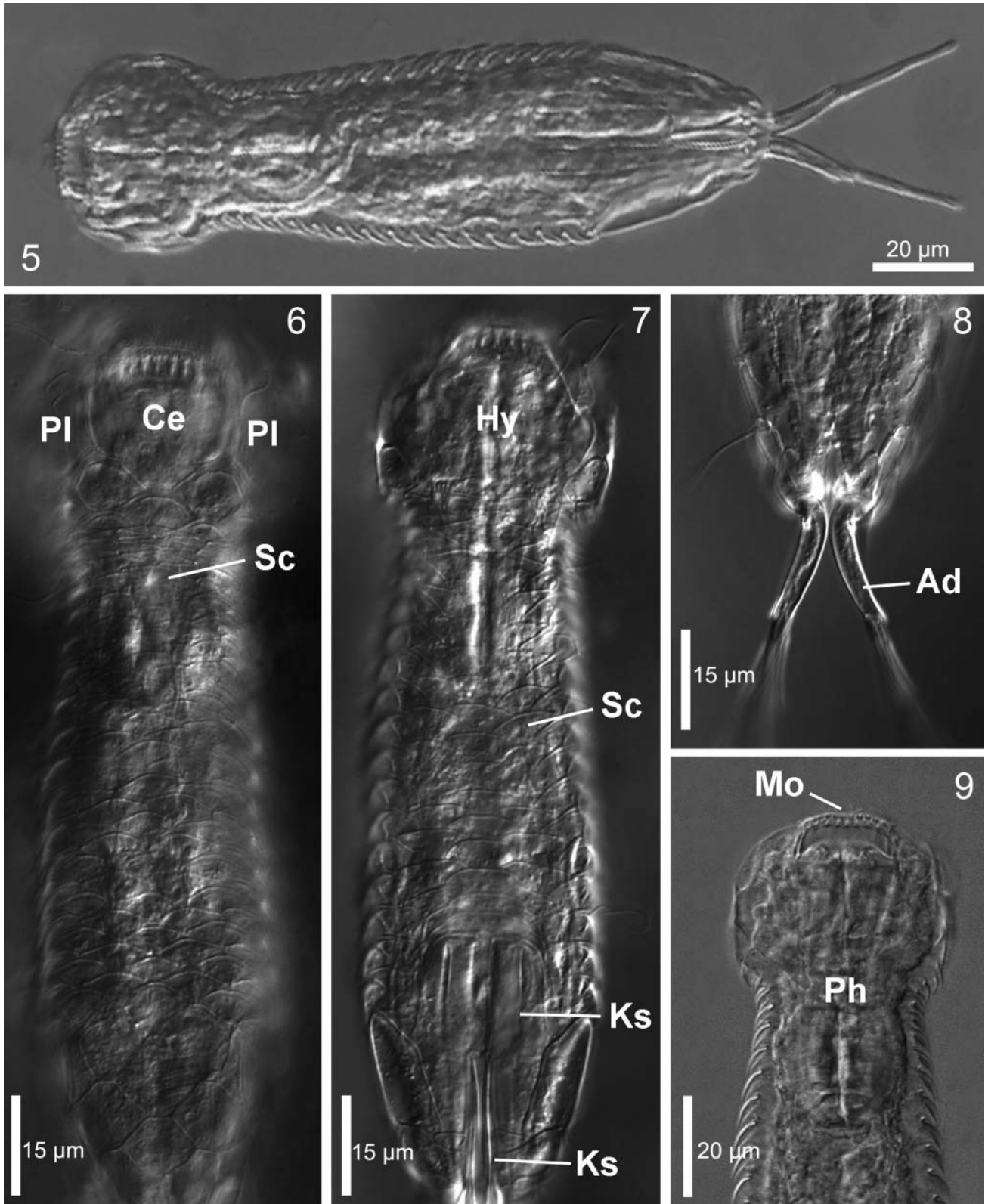


Fig. 5–9. Light micrographs (DIC) of *Cephalionotus kislewskii* gen. et sp. nov. **5**, habitus; **6**, dorsal view of the anterior and trunk region; **7**, ventral view of the anterior and trunk region; **8**, dorsal view of the posterior body end; **9**, internal anatomy of anterior body end. All images refer to the holotype specimen. Ad: adhesive tube, Ce: cephalion, Hy: Hypostomium, Ks: keeled scale, Mo: mouth, Ph: Pharynx, PI: pleura, Sc: spineless scale.

Gen. nov. sp. nov. Araújo et al. (2013); Chaetonotidae sp. Araújo et al. (2016)

Diagnosis. Same diagnosis of the genus.

Etymology. The specific name *kisielewskii* is in homage to Prof. Dr Jacek Kisielewski, a great researcher of Gastrotricha who helped to improve the knowledge about the taxon in Brazil and the world.

Examined material

Holotype. One adult specimen collected from a sandy river in March 2014 at 0.5 m depth, in Preto River, State of Minas Gerais, Brazil, mounted on a glass slide, deposited at the Zoology Museum, University of Campinas, Brazil, under accession number ZUEC GCH 09.

Paratypes. 14 paratype specimens collected from sandy and muddy rivers in March 2014 at 0.5 m depth in Preto River and Veado Pool, State of Minas Gerais, Brazil: nine of them mounted on three glass slides, deposited at the Zoology Museum, University of Campinas, Brazil, under accession numbers ZUEC GCH 10 (two specimens), ZUEC GCH 11 (three specimens), and ZUEC GCH 12 (four specimens). Five of them were mounted for SEM study and are kept at the University of Urbino.

Other material: Three additional specimens were observed alive and are no longer extant. One adult specimen, collected in March 2014 in Veado Pool, State of Minas Gerais, Brazil, prepared for DNA sequencing and no longer extant.

Description. The description is based on both the holotype and six paratypes (see also Table 3). Tenpin-shaped body, 156–204 μm (167 μm in the holotype) in total body length (Figs 2, 4, 5, 16). Marked head, 25–30 μm (30 μm in the holotype) in length, with a weakly three-lobed outline and two paired sensory ciliary tufts (10–15 μm in length). Mouth terminal (14 μm in diameter) (Figs 3, 11, 13). Head delimited from the trunk by a constricted neck (Fig. 5). Body width 28–42 μm (33 μm in the holotype), 20–33 μm (24 μm in the holotype), 30–50 (30 μm) and 13 μm at head (U9), neck (U22), midtrunk (U60), and furca base (U98), respectively. Head wholly covered with very large cephalic plates: a well-developed cephalion (25 μm in length \times 30 μm in width) extends to cover the entire dorsal side of the head, and appears to be almost completely fused with the anterior-most median dorsal scale just posterior to it. Cephalion juxtaposed with three large, rounded spineless scales (respectively 5, 7, and 5 μm in length) lying on the posterior dorsal side of the head (Figs 2, 4, 10, 11). A single rounded pleura (20 μm) on each side of the head, possibly

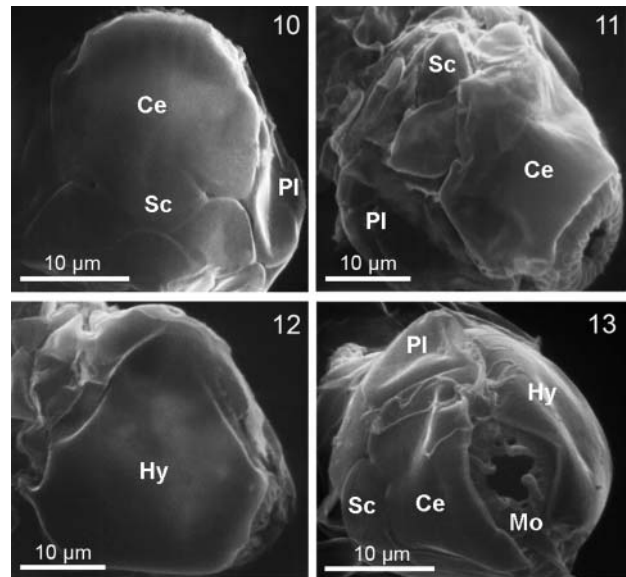


Fig. 10–13. Scanning electron micrographs of *Cephalionotus kisielewskii* gen. et sp. nov. **10**, dorsal view of the head: the arrangement of the cephalion and the three large dorsal head scales are visible, as well as the relationship between cephalion and a pleura; **11**, Dorsolateral view of the anterior end; **12**, ventral view of the head, wholly covered by the large, trapezoidal hypostomium; **13**, frontal view of the head: on the right the cephalion, on the left the hypostomium, and a pleura between them; Images refer to paratypes deposited at University of Urbino. Ce: cephalion, Hy: Hypostomium, Mo: mouth, Pl: pleura, Sc: spineless scale.

deriving from the fusion of an epipleura and a hypopleura, in close contact with dorsolateral edge of the cephalion and ventrolateral edge of the hypostomium (Fig. 13). Hypostomium is an extremely large, trapezoidal plate covering the entire ventral side of the head (21 \times 30 μm) (Figs 4, 7, 12, 13). A single spineless scale (6 \times 5 μm) lies on the head ventrolaterally to the posterior margin of the hypostomium.

Body covered with 9 (7+2) dorsolateral column + 1 ventrolateral column per side and 3 ventral intercalary columns, each of 17–18 spineless, overlapping, hexagonal or rounded scales up to the last third of the body (Fig. 14). First 3–4 scales of each column are smaller (1.5 μm \times 7–8 μm) (Fig. 2) than the subsequent scales (4 μm \times 9–10 μm). On the posterior trunk, 5 columns, each of 3–4 spineless, juxtaposed, large hexagonal scales (8–10 μm \times 10–11 μm) (Figs 2, 6).

Furca, 41–42 μm in length, with a very short base wholly covered with small spineless scales of various shape (Figs 8, 17, 18), and a pair of very long scales (20 μm \times 7 μm). The adhesive tubes, 34–43 μm long (34 μm in the holotype), are particularly thin and trifurcated into three segments along their length (13, 11, and 10 μm , respectively). The diameter of each tube gradually

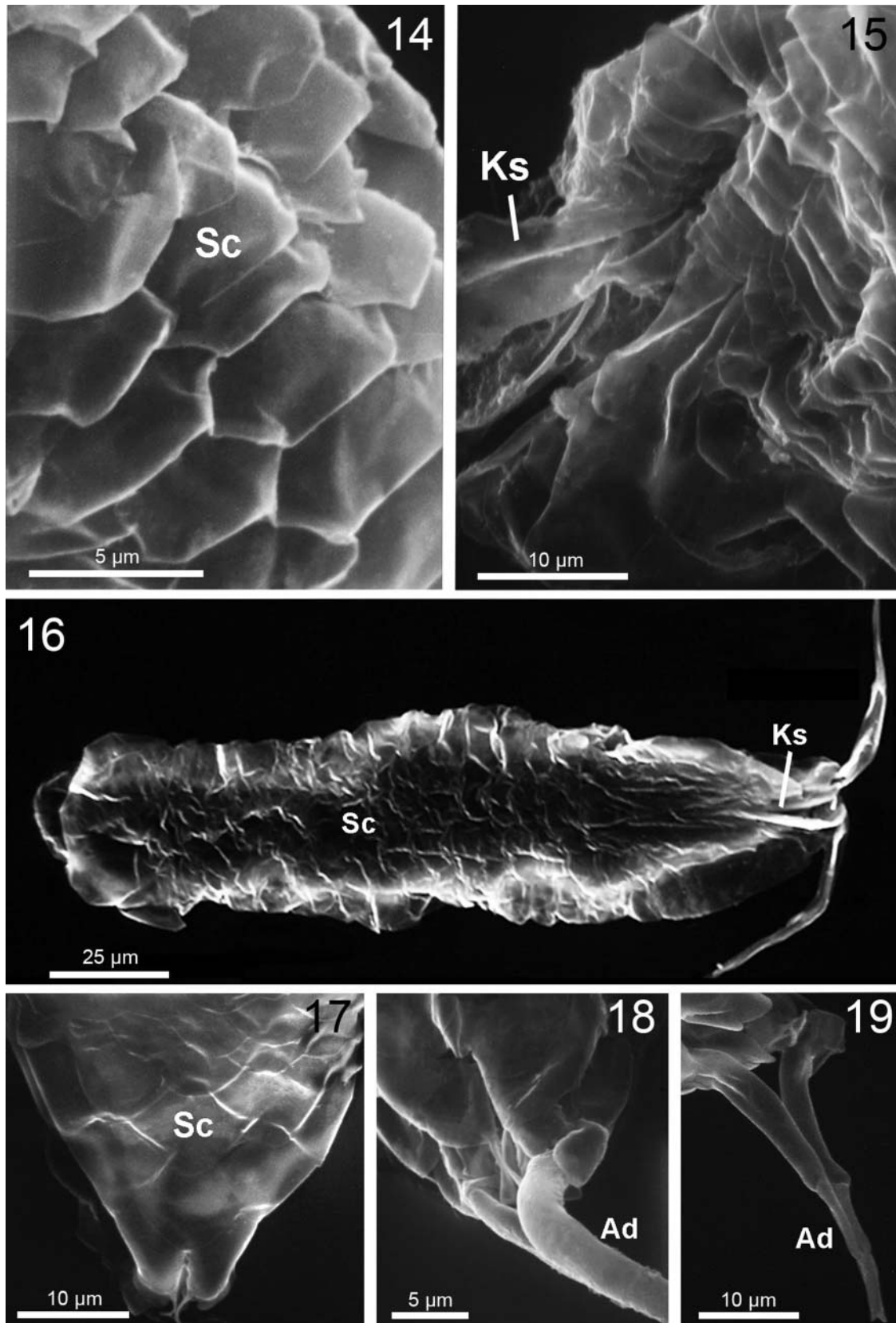


Fig. 14–19. Scanning electron micrographs of *Cephalionotus kisielewskii* gen. et sp. nov. **14**, detail of the scales of the dorsal trunk region; **15**, ventral view of the posterior end of the body; **16**, ventral view of the whole animal; **17**, dorsal view of the posterior trunk region; **18**, dorsolateral view of the furcal base; **19**, detail of the two caudal adhesive tubes, clearly divided into three parts. Images refer to paratypes deposited at University of Urbino. Ad: adhesive tube, Ks: keeled scale, Sc: spineless scale.

Table 3. Morphometric features of *Cephalionotus kisielewskii* gen. et sp. nov. N: total number of measured adult specimens. SD – standard deviation.

Morphometric feature	Range (μm)	Mean	SD	N
Body length	156–204	180 μm	33.9	7
Head length	25–30	27.5 μm	3.5	7
Furca length	41–42	41.5	0.7	7
Adhesive tubes length	36–43	39.5	4.2	7
Pharynx length	51–57	54	4.9	7
Head width	28–42	35	4.2	7
Neck width	20–33	26.5	9.9	7
Midtrunk width	30–50	40	14.1	7
Length/width dorsal neck scale	1.5/7–8	–	–	1
Length/width dorsal trunk scale	4/9–10	–	–	1
Length/width dorsal terminal scale	8–10/10–11	–	–	2
Length/width ventral neck scale	3.3–3.5/10–10.5	3.4/10.25	0.1/0.4	2
Length/width ventral trunk scale	3.7–3.9/10–12	3.8/11	0.1/1.4	2
Length/width terminal trunk scale 1	22/2	–	–	1
Length/width terminal trunk scale 2	40/9	–	–	1
Mouth diameter	14	–	–	1

decreases distally from 3 to 1.5 and 0.7 μm in each part, respectively (Figs 3, 5, 19).

Two paired dorsal sensorial bristles (15 μm in length): one originating from a papilla on the anterior trunk (U30) and the posterior one from a very small triangular hardly visible scale (U66) (Figs 2, 8).

Ventral, large intercalary area covered with 3–5 columns of 15–17 spineless, overlapping scales, rectangular in shape and gradually increasing in size from up to 3.3–3.5 $\mu\text{m} \times 10$ –10.5 μm to 3.7–3.9 $\mu\text{m} \times 10$ –12.5 μm from anterior to posterior, until the last third of the body (U64) (Figs 4, 7). One paired, very large, subrectangular, keeled scale (40 $\mu\text{m} \times 9 \mu\text{m}$), followed by one paired, long, thin (22 $\mu\text{m} \times 2 \mu\text{m}$) keeled, intercalary terminal scale (Figs 4, 7, 15). Some spineless ventrolateral scales of varying sizes, the anterior one much larger than the others (24 $\mu\text{m} \times 7 \mu\text{m}$) (Figs 4, 7, 8).

Two separate longitudinal ciliary bands, not connected anteriorly and extending from the neck (U16) to the middle trunk (U47) were clearly observed in live specimens (Fig. 4).

Large mouth terminal, 12 μm in diameter, wide mouth cavity (Fig. 9). Pharynx, 51–57 μm in length (51 μm in the holotype) with two strong muscular bulbs (respectively, 20–28 $\mu\text{m} \times 20$ –21 μm in the thicker area in the holotype) connected by a short middle region (14 μm in width in the holotype) (Figs 3, 9). No cuticular reinforcement (rods) in the mouth ring.

Sexual organs not observed. Holotype and paratype specimens without eggs, but specimens with an egg (50 μm in length) in the midtrunk dorsal to the gut were observed. In one specimen, four oocytes were also observed, two per side of the posterior intestine.

Phylogeny. The final alignment of the combined dataset yielded 2427 positions (1759 in 18S rDNA and 668 in COI mtDNA). The ML (Fig. 20) and BA (Fig. 21) analyses yielded topologies congruent with each other. Among families analysed in this study, only Neogosseidae appears monophyletic and nested within Dasydytidae, and both families were nested within Chaetonotidae. Most Chaetonotida genera represented in the analyses by at least two species were not monophyletic, except *Stylochaeta* Hlava, 1904 (Dasydytidae), *Halichaetonotus*, *Polymyerurus*, and *Lepidochaetus* (Chaetonotidae) (all supported by high bootstrap and Bayesian posterior probability values, > 0.95 in both cases).

The phylogenetic position of *Cephalionotus kisielewskii* gen. et sp. nov. was stable in both analyses (Figs 20, 21). The new species appeared as the sister-group of *Ichthyidium skandicum* K anneby, Todaro, & Jondelius, 2009 with high bootstrap value (0.99) and low-moderate posterior probabilities (0.87). The clade formed by these two species appeared as a basal branch in the evolution of Chaetonotidae (Figs 20, 21).

Discussion

Cephalionotus kisielewskii gen. et sp. nov. is in many ways a peculiar species of Gastrotricha. It clearly belongs to order Chaetonotida, given its tenpin-like body, with two caudal adhesive tubes, the absence of pharyngeal pores, and the Y-shaped cross-section of the pharyngeal lumen. Moreover, it belongs to the family Chaetonotidae, given the presence of well-developed cephalic plates and cephalic ciliary tufts, a well-developed caudal furca with

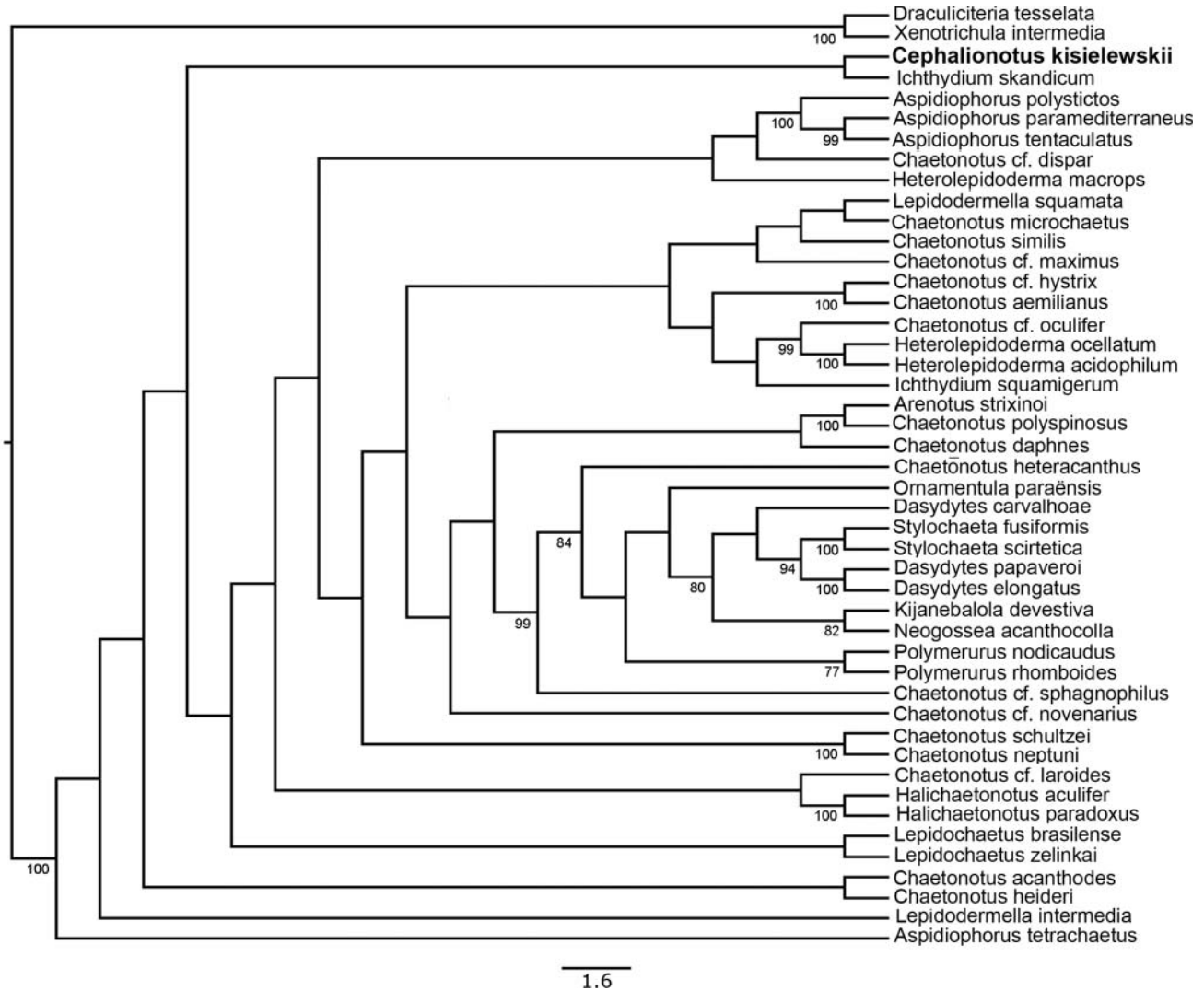


Fig. 20. Phylogenetic relationships of 46 Gastrotricha species inferred from Maximum Likelihood analysis of 18S rRNA and COI mtDNA. Numbers at nodes represents bootstrap support (1000 bootstrap replicates).

very long adhesive tubes, and a ventral locomotory ciliature arranged into two longitudinal bands (Balsamo *et al.*, 2014, 2015; Kisielewski, 1991). However, the species does not appear to fit any existing genus.

The gastrotrich's developed armature with robust scales without spines is highly reminiscent of species of *Lepidodermella*. However, in the latter, the cephalic plates are not as wide as in the new species. *Lepidochaetus brasiliense* and some species of *Chaetonotus* (i.e., *C. heterospinosus*) also show large, spineless scales in some body areas similar to the new species, but always coexisting with spined scales. Species of *Lepidochaetus* and *Polymerurus* have an unusually large cephalion as in *Cephalionotus* gen. nov., but arising posteriorly from the head cuticle.

It is interesting to note apparent similarities between the general habitus of the new species and those of

Draculiciteria tessellata (Chaetonotida, Xenotrichulidae) and species of *Xenodasyys* Swedmark, 1967 (Macrodasysyida, Xenodasyidae Todaro, Guidi, Leasi, & Tongiorgi, 2006), which are psammic species living in marine habitats (Ruppert, 1979; Todaro *et al.*, 2006). They all share a very strong, continuous cuticular armature of spineless scales completely covering the body and particularly strengthened around the head, as well as well-developed furcal appendages. This strongly suggests the effect of a similar psammic lifestyle in producing similar morphological adaptations, even if in different environments.

However, *Cephalionotus kisielewskii* gen. et sp. nov. can be distinguished from all the other Chaetonotidae by the presence of such large cephalic plates that they appear to form a kind of protective 'helmet' around the anterior end: the dorsal side is covered by a very large cephalion that is almost completely fused with the anteriormost

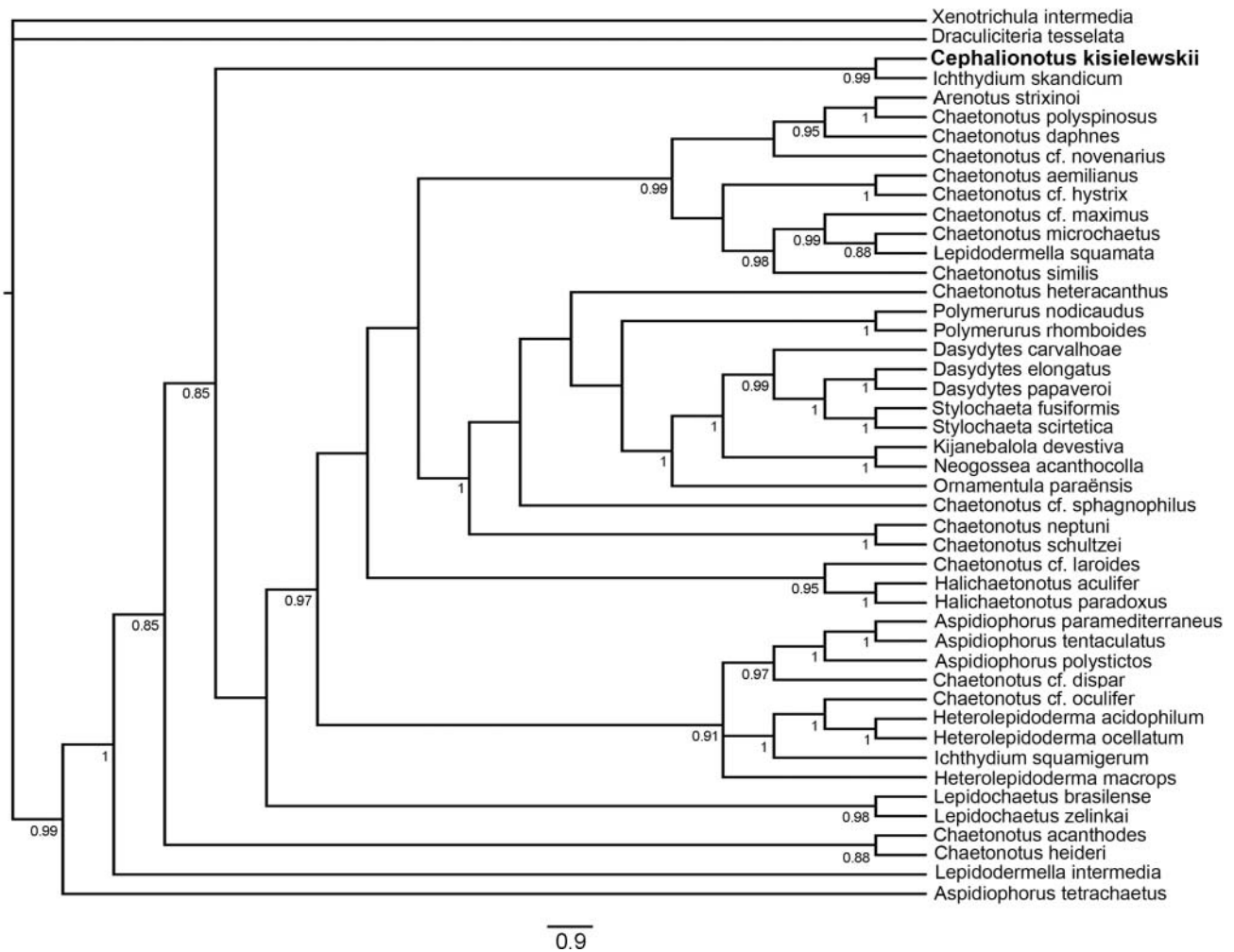


Fig. 21. Phylogenetic relationships of 46 Gastrotricha species inferred from Bayesian inference analysis of 18S rRNA and COI mtDNA. Numbers at nodes represent posterior probabilities.

median scale and is juxtaposed to the three posterior head scales; the lateral sides are covered by a paired pleura, possibly derived from the fusion of an epipleura and a hypopleura; and the ventral side is covered by an extremely large hypostomium. In addition to these unusual features, the new species is also distinguished by the long caudal adhesive tubes divided into three parts, the distinctive scaling of the dorsal and ventral posterior trunk, which features large, juxtaposed spineless scales, and two paired, very large, keeled scales at the ventral end of the body.

The phylogenetic position of *Cephalionotus kisielewskii* gen. et sp. nov. was consistently grouped with *Ichthydium skandicum* in both our ML and BA trees. We interpret this sister group relationship with caution since *I. skandicum* is morphologically quite different, being characterized by a most smooth (featureless) cuticle, except a few scales at the base of the furca (Kånneby, Todaro, & Jondelius, 2009). This apparently incongruent result must

be treated with prudence because cuticular structures may be insufficient to define groups at a low taxonomic level (Kånneby et al., 2013; Kisielewski, 1991). Moreover, the genus *Ichthydium* has been found to be polyphyletic (Kånneby et al., 2013; Kieneke et al., 2008; Kisielewski, 1991). A close relationship between two species with completely different levels of cuticular ornamentation has already been reported for the clade including *Arenotus strixinoi* Kisielewski, 1987, which has a thick, soft, homogeneous cuticular layer, and *Chaetonotus polyspinosus* Greuter, 1917, which has small, spined scales. However, *A. strixinoi* is the only species of this genus, so this result would need a confirmation (Kånneby et al., 2013; Kisielewski, 1987).

At present, our knowledge of the phylogenetic relationships of gastrotrichs at the family and genus levels is still limited, but each year there is improved resolution as more species are added alongside molecular sequence data and improvements in morphological character definitions

(e.g., Guidi, Todaro, Ferraguti, & Balsamo, 2014; K anneby & Todaro, 2015; K anneby *et al.*, 2013; K anneby, Todaro, & Jondelius, 2012; Kieneke, 2010; Kieneke *et al.*, 2008; Kieneke & Ostmann, 2012; Kisielewski, 1991; Todaro *et al.*, 2012b, 2015a; Todaro, Guidi, Ferraguti, & Balsamo, 2012a; Todaro, K anneby, Dal Zotto, & Jondelius, 2011; Todaro, Leasi, & Hochberg, 2014).

Faunistic observations

As pointed out by Todaro *et al.* (2014), 'our knowledge of the biodiversity and phylogeny of the Gastrotricha are far from complete'. This is particularly true of freshwater Gastrotricha from Brazil (Garraffoni *et al.*, 2010). Kisielewski (1987, 1991), in just two papers, described 28 new species, five new genera and one new subfamily. To date, 57 freshwater species of Chaetonotida from Brazil have been reported (Garraffoni & Melchior, 2015; Kisielewski, 1987, 1991). It should be highlighted that this number was obtained through a limited sampling effort, as only a few freshwater habitats have been sampled until now (Ara ujo *et al.*, 2013, 2016; Garraffoni & Melchior, 2015; Garraffoni *et al.*, 2010; K anneby *et al.*, 2013; Kisielewski, 1987, 1991; Todaro *et al.*, 2012b). We also highlight that four of the eight described monospecific genera of Gastrotricha (*Arenotus*, *Undula*, *Ornamentula*, and *Redudasys* Kisielewski, 1987) occur only in Brazil. Thus, the high number of endemic genera and the large number of undescribed species found in Brazilian fresh waters is apparent, even more taking into account the low number of sampled sites. That can be probably related to the enormous size of the country: Brazil features a wide variety of freshwater habitats (ponds, dams, streams, rivers, mangroves, estuaries), offering markedly different geological and abiotic conditions (pH, dissolved oxygen, metal concentrations in sediments, etc.) that can support a great number of species. Therefore, it is not surprising that the number of reported new species (and also new genera) will probably increase quickly in Brazil in the coming years, as many new surveys are planned in the near future to continue the effort to increase taxonomic and biogeographic knowledge of Brazilian Gastrotricha.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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